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NEUROSCIENCE FOREFRONT REVIEW

BUILDING BRAINS IN A DISH: PROSPECTS FOR GROWING CEREBRAL ORGANOIDS FROM STEM CELLS

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Abstract—The recent development of organoid techniques, in which embryonic brain-like tissue can be grown from human or mouse stem cells *in vitro* offers the potential to transform the way in which brain development is studied. In this review, we summarize key aspects of the embryonic development of mammalian forebrains, focussing in particular on the cerebral cortex and highlight significant differences between mouse and primates, including human. We discuss recent work using cerebral organoids that has revealed key similarities and differences between their development and that of the brain *in vivo*. Finally, we outline the ways in which cerebral organoids can be used in combination with CRISPR/Cas9 genome editing to unravel genetic mechanisms that control embryonic development of the cerebral cortex, how this can help us understand the causes of neurodevelopmental disorders and some of the key challenges which will have to be resolved before organoids can become a mainstream tool to study brain development.

Key words: telencephalon, pluripotent stem cells, neural development, tissue engineering.

Contents

INTRODUCTION: MODELING BRAIN DEVELOPMENT

Research on the normal development of the human brain and the etiology of neurodevelopmental diseases faces several major challenges. One rather obvious difficulty is the inaccessibility of the human embryo or fetus, for practical and ethical reasons. Another is the brain's complexity. The human brain contains in the region of 80–90 billion neurons (Azevedo et al., 2009), more than 10 times the number of people alive on the planet today, organized into intricate neuroanatomical structures linked by trillions of connections. Challenges are also posed by the complexity of the mechanisms that control brain development. Considering genetic control alone, for example, brain development depends on the numerous actions and interactions of a large proportion of the 20–25,000 protein-coding genes and unknown numbers of untranslated RNA-coding genes in the human genome (International Human Genome Sequencing Consortium, 2004; Pennisi, 2012). The variability inherent in human populations is a further complication (Frazer et al., 2009). Humans show considerable genetic, epigenetic and environmental variation in factors that modulate the effects of pathogenic events, resulting in significant inter-individual differences in the consequences of a given pathogenic event. While we need to understand the causes of such variability, it makes research on common mechanisms of development and disease harder.

To tackle these problems, many scientists have turned to the use of *in vivo* or *in vitro* biological models that show similarities to aspects of normal or abnormal human brain development, but are simpler, less variable and more readily accessible. In some cases, non-human organisms, most notably the mouse, are used to gain knowledge that might provide mechanistic insights into human development and disease. Using such organisms offers opportunities for controlling inter-individual genetic and environmental variability in

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Abbreviations: dcx, doublecortex; ESCs, embryonic stem cells; IPCs, intermediate progenitor cells; iPSCs, induced pluripotent stem cells; ISVZ, inner subventricular zone; Ngn2, Neurogenin 2; oRG, outer radial glia; OSVZ, outer subventricular zone; PSCs, pluripotent stem cells; RGCs, radial glial cells; SVZ, subventricular zone; TLR3, Toll-like-receptor 3; VZ, vertical zone; ZIKV, Zika virus.

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experiments that would not be possible in humans, but the challenges of studying the development of the intact mouse brain *in vivo* remain significant. Even the mouse brain is highly complex, containing about 70 million neurons (Herculano-Houzel et al., 2006), humans and mice have similar numbers of genes (Waterston et al., 2002; Guenet, 2005) and mouse embryos and fetuses are still relatively inaccessible. Furthermore, as we shall discuss below, although there are great similarities between the brains of humans and mice and the developmental processes that generate them, there are also significant differences that might in some cases complicate or confound attempts to extrapolate between the species.

In vitro models offer considerable advantages due to their accessibility for observation and experimentation involving molecular, cellular or environmental manipulations. Many studies over many decades have used cells and tissues isolated and cultured from the embryonic brains of many species including humans (e.g. Choi and Lapham, 1974; Kim, 1976; Bolz et al., 1990; Molnár and Blakemore, 1991; Price and Lotto, 1996; Hansen et al., 2010, 2013). Such studies have shown that culture systems, in particular organotypic cultures that retain important elements of the tissue's cellular organization, can effectively reproduce key events during brain development, allowing hypotheses on the nature of those events and their regulation to be tested. One limitation of this approach in humans, however, is the inability to manipulate experimentally the genome of the cultured tissues. Whereas in mice material can be derived from the brains of mutant animals, this is clearly unrealistic in humans. An exciting breakthrough in recent years has offered a way of solving this problem. It is now possible to create 3D organotypic cultures that mimic many of the features of the developing brain from pluripotent stem cells (PSCs); these cultured structures have become known as ''organoids".

In the last few years, the development of CRISPR/Cas9 technology has made it much easier to manipulate the genome of human cells (Hockemeyer and Jaenisch, 2016). The confluence of CRISPR/Cas9 and organoid technologies stands to revolutionize our ability to study the genetic control of brain development in humans. Organoids can be used to model human disease in a patient-specific manner, by starting them from stem cells derived from particular individuals, or to study the effects of pathogenic events more generally, as exemplified by their recent high-profile use to study the effects of the Zika virus on early brain development (Garcez et al., 2016; Qian et al., 2016; Cugola et al., 2016; Dang et al., 2016; Nowakowski et al., 2016). The use of organoids coupled with CRISPR/Cas9 is also likely to impact on work in non-human species, streamlining our ability to test the effects of mutations on brain development by lessening the need to generate transgenic animals.

Our focus here is on recent advances in stem cell-derived models in which complex 3D structures with *in vivo*-like properties are generated. First, we shall summarize one of the best-studied and most frequently modeled aspects of *in vivo* brain development in rodents and primates, the formation of the cerebral cortex, before describing the extent to which stem cell-derived cultures can reproduce *in vivo* cortical development. We shall highlight features of primate cortical development not found in the rodent that stem cell-derived cultures might allow us to investigate.

NORMAL CORTICOGENESIS: A COMPARISON OF RODENTS AND PRIMATES

Despite great differences in their sizes, there are numerous similarities in the structure and function of the brains of rodents and primates. They include the conserved laminar structure of the cerebral cortex and its regionalization into major functionally distinct areas with characteristic patterns of connectivity. Many of the fundamental mechanisms of development of these structures are also conserved. In all mammalian species, neurons migrating from the cortical progenitor zones to the overlying developing cortical layers adopt positions related to their birthdate. Each successive generation of newly born projection neurons bypasses earlier-born neurons and settles close to the pial surface immediately below the marginal zone (future cortical layer 1), so that deeper layers are formed before superficial layers, sometimes referred to as an ''inside-out" pattern of development (Angevine and Sidman, 1961; Berry and Rogers, 1965; Rakic, 1974; McConnell, 1995; Tan and Shi, 2013). Neurons arriving in their final laminar positions undergo terminal differentiation, elaborating dendrites and extending axons to establish connections and form cortical circuitry. Within each layer, neurons tend to share similar patterns of gene expression, afferent and efferent connectivity and function across species (Stiles and Jernigan, 2010). But there are many important differences that are more than just differences of scale. These differences appear from early stages of embryonic development.

In the embryos of all mammalian species, neural tube closure is accompanied by its disproportionate anterior expansion to generate the early forebrain from which the left and right cerebral cortices subsequently emerge. In mouse, the production of cortical neurons begins about 10 days after conception and continues for about 8 days (Gillies and Price, 1993; Price et al., 1997; Levers et al., 2001). In humans, as in other primates such as the macaque monkey, cortical neurogenesis occurs over many weeks, starting at about 35 days post-conception and finishing about 3 months later (Rakic, 1974; Bystron et al., 2008; Bayatti et al., 2008). One very striking difference between the events that generate the cortex of primates and rodents is the time it takes progenitor cells to go through their cell cycles. Primate cell cycle times, which are very similar in human and non-human primates, can be up to five times longer than in rodents at corresponding developmental stages (Takahashi et al., 1995; Kornack and Rakic, 1998; Haydar et al., 2003; Lukaszewicz et al., 2005; Breunig et al., 2011). These differences in the neurogenic period and cell cycle times are likely to be extremely important in explaining differences between primates and rodents not only because they influence the numbers of neurons generated but also

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because the length of the cell cycle appears to influence their laminar phenotypes (Dehay and Kennedy, 2007; Pilaz et al., 2009). An important question is whether stem cell-derived cortical tissue produced in culture replicates the species-specific cell cycle times found *in vivo*.

At the earliest stages of mammalian forebrain formation, neuroepithelial progenitor cells undergo divisions at the neural tube's inner surface (also known as the apical or ventricular surface) to generate two new progenitors (Fig. 1). These divisions, known as symmetric or proliferative divisions, expand the pool of progenitors. The population of early symmetrically dividing neuroepithelial cells soon transforms and diversifies. Prominent among the new cell types are radial glial cells (RGCs). It had been known for decades that RGCs, whose long processes span the neuroepithelium, provide guidance for migrating neurons (Levitt and Rakic, 1980; Rakic, 1988). Despite having morphological and molecular features associated with glial cells, RGCs are also progenitors capable of regenerating themselves and generating other types of progenitors, neurons and glial cells (Malatesta et al., 2000; Noctor et al., 2001; Tan and Shi, 2013).

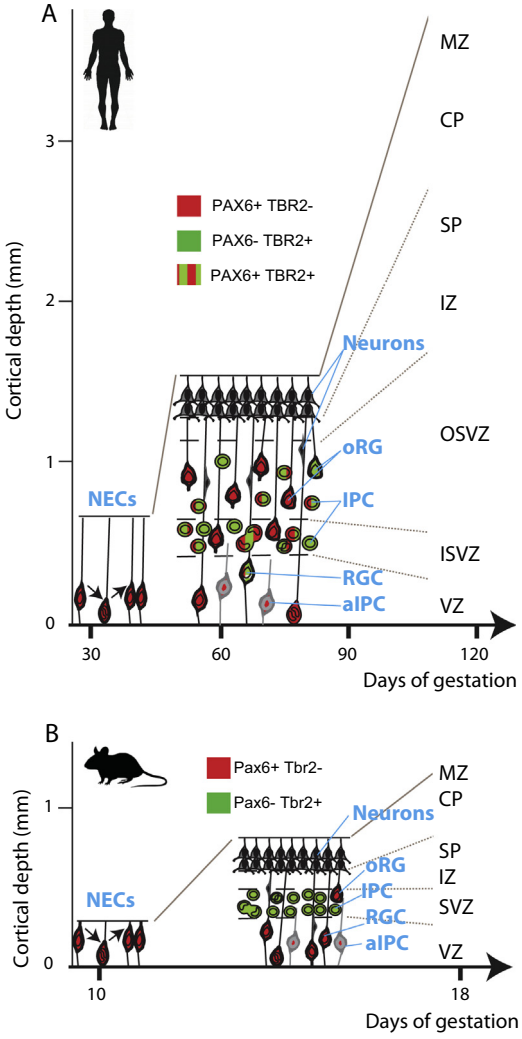

Fig. 1. Comparison of human and mouse cortical development. Diagrams of sections through the depth of the developing cortex of (A) humans and (B) mice showing the major progenitor types and whether they express the transcription factors PAX6/Pax6 and/or TBR2/Tbr2. Mouse corticogenesis occurs over a much shorter period of time than human corticogenesis. Abbreviations: MZ, marginal zone; CP, cortical plate; SP, subplate; IZ, intermediate zone; OSVZ, outer subventricular zone; ISVZ, inner subventricular zone; VZ, ventricular zone; SVZ, subventricular zone; NECs, neuroepithelial cells; oRG, outer radial glia; IPC, intermediate progenitor cell; RGC, radial glial cell; aIPC, apical intermediate progenitor cell.

The progenitor cells that divide at the neural tube's inner surface are often referred to as apical progenitors and they form a layer known as the ventricular zone (VZ; Fig. 1). In the VZ, progenitors undergo interkinetic nuclear migration: their nucleus moves radially through the cytoplasm such that mitosis occurs at the apical surface and S-phase at the opposite, basal edge of the VZ. As forebrain development progresses, an increasing proportion of RGCs divide asymmetrically to produce other cell types (Noctor et al., 2001, 2004; Haydar et al., 2003; Tan and Shi, 2013; Florio and Huttner, 2014; Paridaen and Huttner, 2014; Rakic, 2009). Some daughter cells migrate radially to the pial surface to differentiate into neurons or, later in development, glia (Levers et al., 2001). Many become a new type of progenitor that, instead of dividing at the apical surface of the VZ, divides in a region superficial to it called the subventricular zone (SVZ). These progenitors are referred to as intermediate progenitor cells (IPCs) (Fig. 1; Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004).

At this stage, however, major differences emerge between the SVZs of rodents and primates. In rodents, IPCs in the SVZ divide mainly symmetrically to generate two neurons, which migrate into the developing cortex (Farkas and Huttner, 2008). Progenitors in the primate SVZ divide repeatedly and asymmetrically to expand this zone greatly compared to that of rodents (Smart et al., 2002; Dehay et al., 2015). Primates develop two subventricular proliferative layers, the inner and outer subventricular zones (ISVZ and OSVZ respectively) (Fig. 1A; Smart et al., 2002; Lukaszewicz et al., 2005; Zecevic et al., 2005; Fietz et al., 2010; Hansen et al., 2010; Florio and Huttner, 2014). The ISVZ contains mainly IPCs, which are equivalent to IPCs in the rodent SVZ. The OSVZ, on the other hand, contains progenitors with similar molecular expression profiles and neurogenic properties to RGCs in the VZ, except that they lack processes linking them to the apical surface (Fietz et al., 2010; Hansen et al., 2010; Reillo et al., 2011; Hevner and Haydar, 2012; Florio and Huttner, 2014). These OSVZ progenitors have become known as outer radial glia (oRG; Fig. 1). They undergo proliferative divisions and self-renewing asymmetric divisions to generate one oRG daughter cell and one IPC that can proliferate further (Fietz et al., 2010; Hansen et al., 2010; Florio and Huttner, 2014). Although oRG have been observed in the rodent SVZ, they account for only a minute fraction of the SVZ progenitors whereas they constitute about half of all progenitors present in the primate OSVZ (Dehay et al., 2015). The OSVZ is the major source of neurons for the superficial (or supragranular) cortical layers; these layers, which carry out critical functions in intracortical integration, show

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greater enlargement than other cortical layers in primates (Smart et al., 2002; Lukaszewicz et al., 2005; Dehay et al., 2015). In fact, the OSVZ is the major germinal zone of the developing primate cerebral cortex and, from mid-corticogenesis onward, contains most of the cortical progenitors (Smart et al., 2002; Lukaszewicz et al., 2005; Fietz et al., 2010; Hansen et al., 2010; Betizeau et al., 2013; Dehay et al., 2015). Additional cells types are found in the VZ and SVZs of rodents and primates, e.g. a small population of apical IPCs (aIPCs; Fig. 1; Gal et al., 2006; Tan and Shi, 2013) but the full extent of this heterogeneity remains unclear, particularly in primates (Hansen et al., 2010; Fietz and Huttner, 2011; Betizeau et al., 2013; Dehay et al., 2015; Pfeiffer et al., 2016).

In the mouse cortex, apical progenitors are distinguished by their expression of the transcription factor Pax6 and apical progenitors that give rise to IPCs transiently express the proneural transcription factor Neurogenin 2 (Ngn2) (Britz et al., 2006). Pax6 is expressed neither in IPCs, which are characterized by their expression of the transcription factor Tbr2, nor in postmitotic neurons, which express Tbr1. Thus, sequential Pax6 → Ngn2 → Tbr2 → Tbr1 expression correlates with the transition of apical progenitors to IPCs to postmitotic neurons (Englund et al., 2005; Telley et al., 2016). In primate corticogenesis, however, Pax6 (PAX6 in humans) is expressed by progenitors in the VZ, ISVZ and OSVZ (Fietz et al., 2010; Betizeau et al., 2013; Florio and Huttner, 2014) with many progenitors co-expressing both Pax6/PAX6 and Tbr2/TBR2 (Fig. 1).

The descriptions and comparisons above concern the development of the excitatory projection neurons of the cortex, which transmit signals over relatively long distances. This is only one of the two major classes of cortical neurons, the other being the short-range GABAergic inhibitory interneurons that modulate the activity of cortical circuits locally. There may be differences between rodents and primates in the processes that generate these inhibitory interneurons. In rodents, cortical interneurons originate from distant subcortical germinal domains, mostly in the ganglionic eminences, from where they follow tangential migratory routes to reach the developing cortex (Gelman and Marin, 2010). In primates, several studies have suggested that, while many interneurons also originate subcortically, a significant fraction is produced in the progenitor layers of the cortex itself during the second half of corticogenesis (Zecevic et al., 2005; Radonjić et al., 2014). This issue is not resolved, however, since a study by Hansen et al. (2013) found no evidence of interneuron production in the primate cortical wall.

All of this work on *in vivo* development provides a rich dataset against which to test the potential of stem cell-derived systems to reproduce in culture the processes and mechanisms that occur *in vivo*. It raises many important questions. Can species-specific processes be replicated in a dish? Do progenitors have much longer cell cycle times in stem cell-derived cultures from humans than from mice? Can oRG and the equivalent of the oRG-containing OSVZ be generated from human stem cells? Are the species-specific gene expression patterns associated with different classes of progenitor reproduced in stem cell-derived *in vitro* systems? We shall consider the extent to which such questions are answered by existing research and highlight important areas for further study.

CAN WE USE PSCS TO MODEL CORTICOGENESIS?

PSCs have been used to study molecular mechanisms that control many types of cellular differentiation (Martello and Smith, 2014). There are two major types of PSC, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). ESCs are derived from blastocyst stage mouse or human embryos and iPSCs are made by reprogramming differentiated cells from adult tissue to a pluripotent state. iPSCs were first described ten years ago and offer the unique advantage that they can be obtained from any individual including, for example, those affected by neurodevelopmental diseases (Takahashi and Yamanaka, 2006).

Early protocols for promoting neural differentiation of PSCs involved allowing the cells to form large multicellular aggregates known as embryoid bodies. However, following the demonstration that 3D-aggregation is not essential for efficient neural differentiation (Ying et al., 2003), a number of highly efficient protocols for 2D monolayer differentiation of ES cells into cortical neurons were developed (Gaspard et al., 2008; Chambers et al., 2009; Shi et al., 2012). These 2D cultures contain progenitor cells similar to those seen in the developing cortex which show correct apico-basal polarity and undergo interkinetic nuclear migration. They show a degree of spatial information – cells become organized into rosette-shaped structures, with radial glial progenitors located at the center and oRG-like progenitors located toward the periphery of the rosettes (Gaspard et al., 2008; Chambers et al., 2009; Shi et al., 2012; Otani et al., 2016). RGCs in 2D cortical rosette cultures grown from macaque PSCs divided with a cell cycle length of around 35 h at 32 days in culture (Otani et al., 2016), compared to the 23 h which has been reported for macaque RGCs at E40 *in vivo* (Kornack and Rakic, 1998). Equivalent cultures derived from human PSCs showed an average cell cycle time of around 45 h (Otani et al., 2016). Interestingly, time-lapse imaging of both macaque and human PSC-derived rosette cultures showed a large range of cell cycle times, with some cells dividing in under 12 h and others taking more than 100 h (Otani et al., 2016). Human PSC-derived RGCs in 2D cultures continued proliferation over a longer period than those derived from macaque PSCs, suggesting that a protracted expansion phase contributes to the increased size of the human cortex (Otani et al., 2016). 2D rosette cultures produce both deep and superficial layer projection neurons that have mature electrical properties and form functional synapses (Shi et al., 2012). Neuronal types characteristic of all six cortical layers have been successfully generated in 2D-cultures of human PSCs (Espuny-Camacho et al., 2013), but they do not form the characteristic layers found normally in the cortex.

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Nonetheless, pyramidal cortical neurons derived in 2D-culture from mouse ES cells were able to integrate into damaged mouse cortex, where they established functional connections (Michelsen et al., 2015). 2D cultures clearly reproduce several aspects of normal cortical development but, as they lack the 3D organization and tissue architecture of the normal cerebral cortex, developmental processes that depend upon this are unlikely to occur as normal. Therefore, it seems likely that 3D-cultures should resemble the developing cortex more closely and therefore make more accurate models. A comparison of some of the key strengths and weaknesses of 2D and 3D cultures is shown in Table 1.

A growing body of work over the last few years has shown that PSCs grown under appropriate conditions can give rise to 3D organ rudiments, known as organoids (reviewed by Sasai, 2013; Lancaster and Knoblich, 2014; Huch and Koo, 2015). Organoids contain a variety of specialized cell types, whose arrangement and behaviors resemble those seen in the cognate embryonic tissue (Lancaster and Knoblich, 2014). The first organoids to be reported were derived from intestinal stem cells and comprise intact crypt-villus structures, usually referred to as miniguts (Sato et al., 2009), and these are probably the best characterized type of organoid described to date (reviewed by Sato and Clevers, 2013). Protocols have now been described for the derivation of organoids corresponding to a wide range of tissue types including optic cups (retina) (Eiraku et al., 2011), adenohypophysis (the neural part of the pituitary) (Suga et al., 2011), neural tube (Meinhardt et al., 2014) and early cerebellum (Muguruma et al., 2015). Most relevant here,

Table 1. Comparison of some key strengths and weaknesses of 2D versus 3D cultures

Feature	2D	3D	References
Gives rise to wide range of neural progenitor types and cortical neurons	Yes	Yes	1–11
Structural organization of cultured cells/tissue	Poor – cannot fully reproduce complexity of 3D tissue	Good – more closely resembles *in vivo* tissue	1–11
Ease of visualizing and tracking individual cells	Excellent	Can be done, but more technically challenging	1, 3-9, 11
Availability of nutrients to cultured cells/tissue	Excellent	May require additional measures, such as spinning bioreactor	4,5,8
Requirement for Matrigel (a potential source of experimental variation)	Not required	Usually required, although may be possible to replace with synthetic hydrogels	1–11
Ease of experimental manipulation	All cells directly accessible by drugs/compounds added to medium	Accessibility of internal cells in organoids may be reduced. Localized application of substances (eg on microbeads) likely easier.	

References: 1. Gaspard et al. (2008) 2. Espuny-Camacho et al. (2013) 3. Michelson et al., (2016) 4. Shi et al. (2012) 5. Otani et al. (2016) 6: Nasu et al. (2012) 7: Kadoshima et al. (2013) 8: Lancaster et al. (2013) 9: Eiraku et al. (2008) 10: Mariani et al. (2012) 11:Paşca et al., (2015).

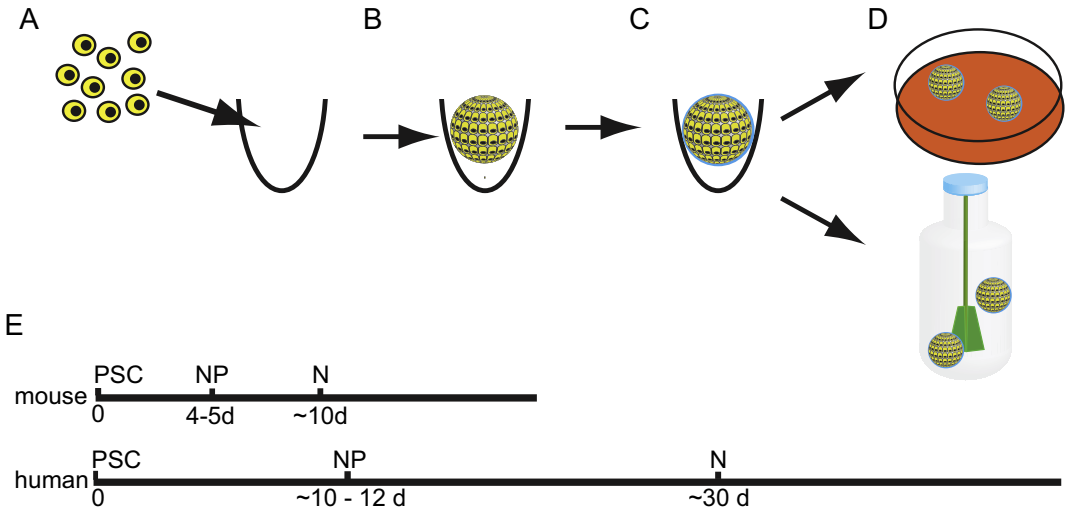

Fig. 2. Outline protocols for growing cerebral organoids from PSCs. (A) PSCs are placed in individual wells of a 96-well non-adherent cell culture plate. (B) PSCs are allowed to aggregate. (C) Matrigel (blue) is added to PSC aggregates. (D) Organoids are transferred to differentiation medium and cultured in either non-adherent Petri dishes (top) or a spinning bioreactor (bottom). (E) Mouse and human PSCs follow species-specific timelines of differentiation. Abbreviations: PSC, pluripotent stem cell; NP, neural progenitor; N, neuron.

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cerebral organoids, which resemble embryonic cerebral cortex, have been derived from both mouse and human PSCs (Eiraku et al., 2008; Nasu et al., 2012; Lancaster et al., 2013; Paşca et al., 2015).

Cerebral organoids are most commonly made by allowing PSCs to form aggregates of a few thousand cells in low-adhesion culture plates (outlined in Fig. 2). Protocols vary, but common features include inhibition of SMAD signaling to enhance neural induction (Chambers et al., 2009; Lancaster et al., 2013) and inhibition of Wnt signaling to promote the induction of forebrain fate (Watanabe et al., 2005; Nasu et al., 2012; Kadoshima et al., 2013). Published protocols for growing human brain organoids have recently been reviewed in detail by Kelava and Lancaster (2016). The simplicity of these protocols appears consistent with the idea that anterior forebrain fates arise by default, so long as posteriorizing signals (including Wnts) are suppressed (Wilson and Houart, 2004).

CEREBRAL ORGANOIDS EXHIBIT MANY OF THE CHARACTERISTICS OF EMBRYONIC CEREBRAL CORTEX

Cerebral organoids made from either mouse or human PSCs demonstrate key hallmarks of normal forebrain development (summarized in Table 2). Mouse cerebral organoids contained neural progenitor cells organized in a similar way to that seen *in vivo*. Cells comprising the innermost layer of the organoids (adjacent to a fluid-filled lumen) developed morphological and molecular features of RGCs. The use of live imaging allowed tracking of the behavior of individual GFP-labeled RGCs within organoids. This showed that RGCs exhibited interkinetic nuclear migration, underwent S-phase in the basal region of the proliferative zone and mitosis in the apical region, similar to the behavior of RGCs *in vivo* (Nasu et al., 2012). Pax6 was expressed by RGCs in organoids; Ngn2- and Tbr2-expressing cells were located progressively superficial to the bulk of the Pax6-expressing progenitors, indicating the presence of appropriately located IPCs; Tbr1-expressing cells were observed in the outer layers, indicating the presence of appropriately located postmitotic neurons (Fig. 3A) (Nasu et al., 2012). This strongly suggests that the Pax6 → Ngn2 → Tbr2 → Tbr1 expression sequence found in mouse cortex can be reproduced in cortical organoids.

Neurons in different cortical layers express specific markers that allow us to identify them (Fig. 3B). In mouse cerebral organoids, specific subtypes of cortical neurons were produced in the same temporal order as found *in vivo* (Nasu et al., 2012). The earliest-born cortical neurons *in vivo* are the Cajal-Retzius (CR) cells, which are found in layer 1 and express Calretinin and Reelin (Marin-Padilla, 1983; Derer and Derer, 1990; Meyer et al., 1999). Reelin + Calretinin + cells, likely to be the *in vitro* equivalents of CR cells, were formed early in cerebral organoids (Fig. 3C). This was followed by the generation of neurons expressing Tbr1 and Ctip2, which mark deep layer neurons, and then neurons expressing Cux1, which marks superficial layer neurons (Fig. 3D). Although Ctip2 + /Tbr1 + and Cux1 + cells were born in the correct order, the Cux1 + cells did not migrate outward to form a clear upper layer superficial to the Ctip2 + /Tbr1 + cells (Fig. 3D). Cux1 + cells remained deep, indicating that the methods used did not allow migration to progress fully through all of its later stages (Nasu et al., 2012).

Cerebral organoids derived from human PSCs also contained a VZ, consisting predominantly of PAX6 and SOX2-expressing RGC-like cells that exhibited interkinetic nuclear migration and underwent mitosis at the apical edge, as shown by live imaging of individual labeled cells within organoids (Kadoshima et al., 2013; Lancaster et al., 2013). Cell cycle times of progenitor cells in 3D organoid cultures have not yet been reported, but we do know that mouse and human cerebral organoids each grow according to species-specific time lines. For example it takes around 6–8 days for neurons to appear in mouse organoids, but closer to four weeks in human (Eiraku et al., 2008; Nasu et al., 2012; Lancaster et al., 2013). Similarly, it takes around two weeks for cortex-like structures to emerge in mouse organoids, but more than 10 weeks in human, consistent with the much longer neurogenic period in human embryos (Nasu et al., 2012; Lancaster et al., 2013).

As described above, a major difference between mouse and human embryonic cortex is the presence of substantial numbers of PAX6 + /SOX2 + /TBR2- oRG progenitor cells in the human SVZ. By 12–13 PCW (post conception weeks), the human SVZ is much thicker than the VZ and contains large numbers of

Table 2. Summary of key hallmarks of cortical development shown by cerebral organoids grown from mouse and human PSCs. References: 1: Nasu et al. (2012) 2: Kadoshima et al. (2013) 3: Lancaster et al. (2013) 4: Eiraku et al. (2008) 5: Mariani et al. (2012) 6: Paşca et al., (2015) ND: not determined

Feature	Mouse	Human	Refs
Radial glial cells present, show interkinetic nuclear migration and mitoses at ventricular edge	Yes	Yes	1-6
Presence of outer radial glia (oRG)	Few, if any	Yes	2,3
Time until neurons formed	6–8 days	> 4 weeks	1-6
Time until cortex-like structures form	~2 weeks	~10 weeks	1-4
Clear separation between progenitor cells and neurons	Yes	Yes	1-6
Formation of Cajal-Retzius cells	Yes	Yes	1,2,3,4,6
Formation of deep layer (early born) neurons	Yes	Yes	1,2,3,4,6
Formation of superficial layer (late born) neurons	Yes	Yes	1,2,3,4,6
Lamination – clear separation of deep and superficial layer neurons	No	Yes	1,2,3,6
Expression of cortical area-specific markers	ND	Yes	2,3

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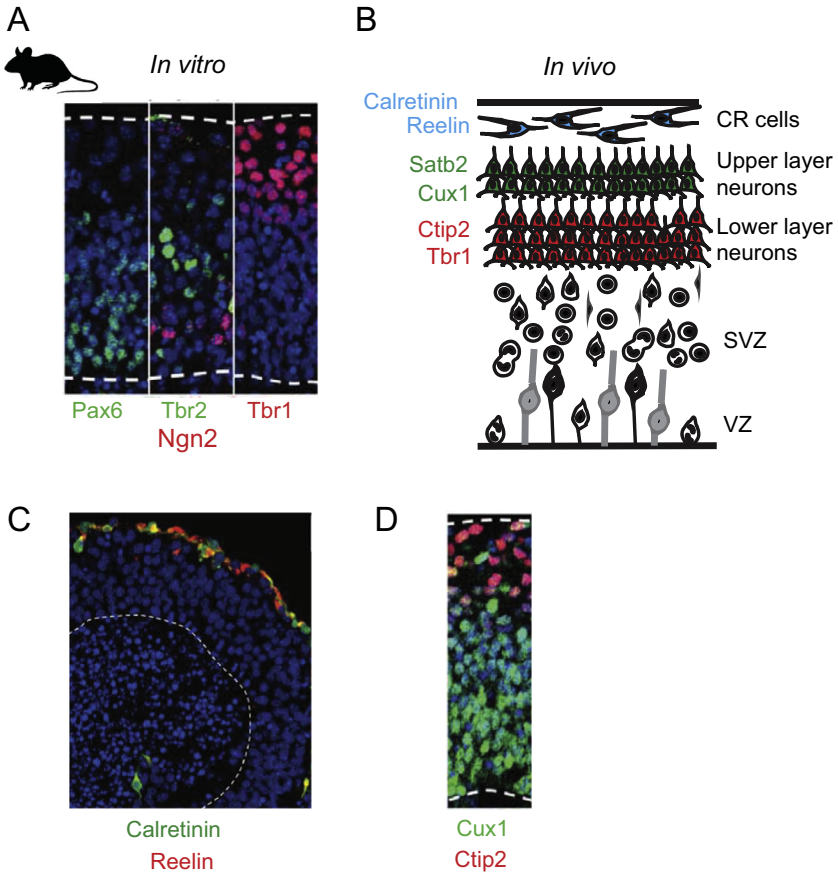

Fig. 3. Cortical organoids in mice. (A) Sections taken from cerebral organoids derived from mouse ES cells after 15 days in culture, showing expression of Pax6, Tbr2, Ngn2 and Tbr1. Dotted lines indicate the edges of the neuroepithelium. Pax6 expressing cells are located apically, Tbr2- and Ngn2-expressing cells are located basal to these, and Tbr1-expressing cells are found at the outer edge of the neuroepithelium. (B) Cartoon illustrating the expression of layer-specific markers in embryonic cerebral cortex. SVZ: subventricular zone, VZ: ventricular zone, CR: Cajal-Retzius cells. (C) Expression of Cajal-Retzius cell markers calretinin and reelin at the outermost edge of a mouse cerebral organoid. (D) Expression of layer-specific cortical markers in mouse cerebral organoids. Both Cux1+ (upper layer) and Ctip2+ (deeper layer) neurons are present, but do not laminate correctly – Cux1+ neurons are located deep to the earlier-born Ctip2+ population. Panels (A) (C) and (D) are modified from Nasu et al. (2012) with permission.

PAX6+/SOX2+/TBR2- oRG cells (Fig. 4A; Hansen et al., 2010). A clear SVZ region was present in similarly-aged human cortical organoids, containing PAX6+/SOX2+/TBR2- cells, very likely corresponding to oRG (Fig. 4B; Kadoshima et al., 2013; Lancaster et al., 2013). Like oRG *in* vivo, these cells had a basal process, but not an apical one and many fewer of them were found in mouse organoids. oRG showed patterns of division similar to those reported in human brain slice cultures (Kadoshima et al., 2013; Lancaster et al., 2013). Taken together, these analyses clearly indicate that a cell population analogous to oRG is present in human organoids, although there appear to be substantially fewer of them than are found in equivalent-aged cortex *in vivo* (Fig. 4A, B) perhaps suggesting that the organoids may develop at a slower rate than the embryonic cortex. Given the significance of oRG in development of the human cerebral cortex and their proposed importance in driving increases in brain size during evolution, it will be important in future studies to ascertain the extent to which the properties and behaviors of organoid oRG resemble their *in vivo* counterparts. In particular, it will be important to establish whether organoid oRGs can give rise to very large numbers of cortical neurons.

In human cerebral organoids, neurons expressing markers of superficial layer neurons (SATB2, CUX1, BRN2) were born later than neurons expressing deeper layer markers CTIP2 or TBR1 and migrated through them to form a more superficial layer (Fig 4C). This suggests that, in human organoids, the migration of neurons to appropriate relative depths based on their birthdates recapitulated that seen in the embryonic cerebral cortex *in vivo* (Kadoshima et al., 2013; Lancaster et al., 2013). Birthdating analysis showed that later born neurons migrated outward past earlier-born neurons (Kadoshima et al., 2013), but a full separation of cortical neurons into morphologically, molecularly and functionally distinct layers has not yet been reported. It seems surprising that the migration of neurons to form cortical layers is reproduced more faithfully in human organoids than in mouse, suggesting that factors needed for full migration are absent from the mouse cultures, either due to differences in culture conditions or to intrinsic differences between mouse and human cells. Organoids

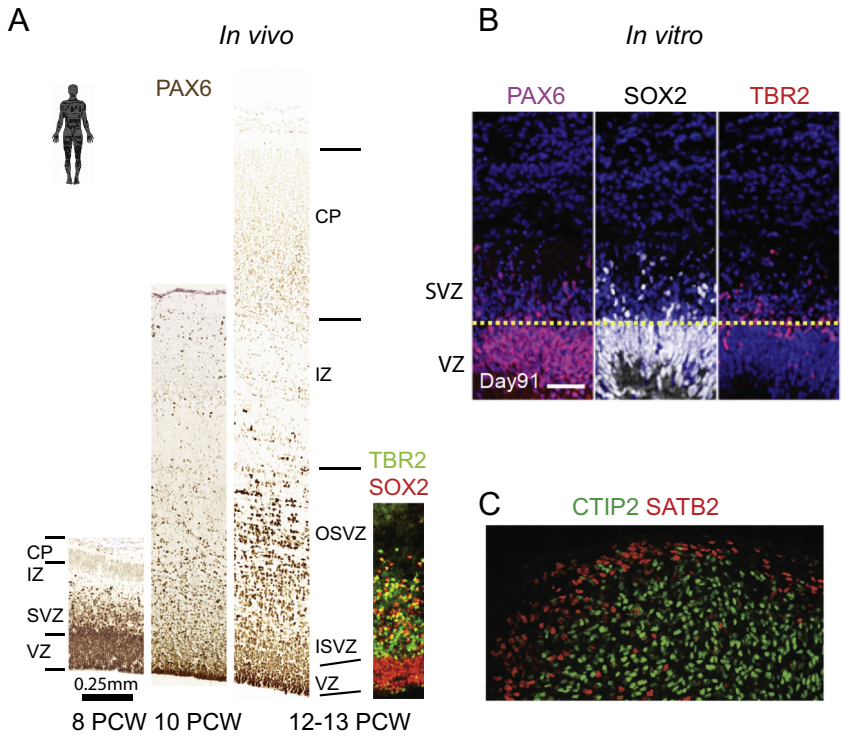

Fig. 4. Cortical organoids in humans. (A) Images of PAX6 expression in human cortices at 8, 10, and 12 post-coital weeks (PCW) were generated using material from the Human Developmental Biology Resource (www.hdbr.org) as part of the HuDSeN (Kerwin et al., 2010) human gene expression spatial database (http://www.hudsen.org) based at Newcastle University. PAX6 is expressed in the ventricular zone (VZ) and subventricular zone (SVZ) at eight PCW. The SVZ divides into an outer and an inner subventricular zone (OSVZ and ISVZ), both of which continue to express PAX6. Data on expression of TBR2 and SOX2 in the VZ, ISVZ and OSVZ at 13 PCW are from Hansen et al., 2010. (B) Expression of PAX6, SOX2 and TBR2 in human cerebral organoids, PAX6+ and SOX2+ cells are seen in the SVZ, consistent with the presence of oRG. Dashed line indicates the boundary between the VZ and the SVZ. Data reproduced from Kadoshima et al., 2013, with permission. (C) Expression of layer-specific cortical markers in human cerebral organoids. Neurons expressing the upper layer marker SATB2 are found located superficial to those expressing the deep layer marker CTIP2, as seen in the cortex *in vivo* (Fig 3C). Reproduced from Lancaster et al., 2013, with permission.

may provide a useful model to identify factors required for full cortical lamination, through testing the ability of candidates to enhance or to fully restore normal patterns of neuronal migration.

The mature cerebral cortex is regionally organized, with different regions having different functions. This regionalization is initiated during embryogenesis by secreted morphogens that are produced by signaling centers surrounding the developing forebrain (reviewed by O'Leary and Sahara, 2008; Hoch et al., 2009; Borello and Pierani, 2010). One such signaling center, located at the rostral (anterior) pole of the cortex, secretes several FGF proteins, such that FGF activity is high rostrally and low caudally. This FGF gradient sets up gradients of expression of several transcription factors, including COUP-TF1, SP8 and OTX2, which contribute to patterning of the emerging cortex into specific regions by controlling expression of region-specific transcription factors, including AUTS2, TSHZ2 and LMO4 which are specifically expressed in prefrontal, occipital and frontal/occipital cortex respectively. Interestingly, COUP-TF1, SP8 and OTX2 were reported to be frequently expressed in gradients in human cerebral organoids (Fig. 5A, B; Kadoshima et al., 2013). Strikingly, some human organoids expressed COUP-TF1 and SP8 in countergradients (Fig 5A) like those seen in the embryonic cortex *in vivo* (Fig. 5C, summarizes expression patterns as seen in mouse cortex). The level of pERK, a molecule activated in the FGF signaling pathway, was highest in the region where COUP-TF1 expression was lowest (Fig. 5B, white bracket) indicating that FGF signaling was regionally active and suggesting that it may underlie the formation of Sp8 and COUP-TF1 expression gradients. In support of this idea, adding FGF8 to organoid cultures led to increased Sp8 and decreased COUP-TF1 expression (Kadoshima et al., 2013). The area-specific marker genes AUTS2, TZH2 and LMO4 are each expressed in restricted domains in human organoids (Fig. 5D; Lancaster et al., 2013) further suggesting that the organoids reproduce some degree of cortical arealization. These findings raise a particularly interesting question about cerebral organoids – do they contain analogs of the signaling centers that surround the developing forebrain *in vivo*? There is evidence that a structure analogous to the cortical hem, a signaling center located at the medial edge of the cortex, is present in both mouse and human organoids. A narrow strip of cells was found at one edge of organoid cortex which expressed transcription factors that mark the cortical hem *in vivo* (Nasu et al., 2012; Kadoshima et al., 2013). It will be very interesting to determine whether this hem-like tissue expresses the Wnt and BMP signaling molecules normally produced by the cortical hem and, if so, whether neighboring cortical cells respond to them during organoid development.

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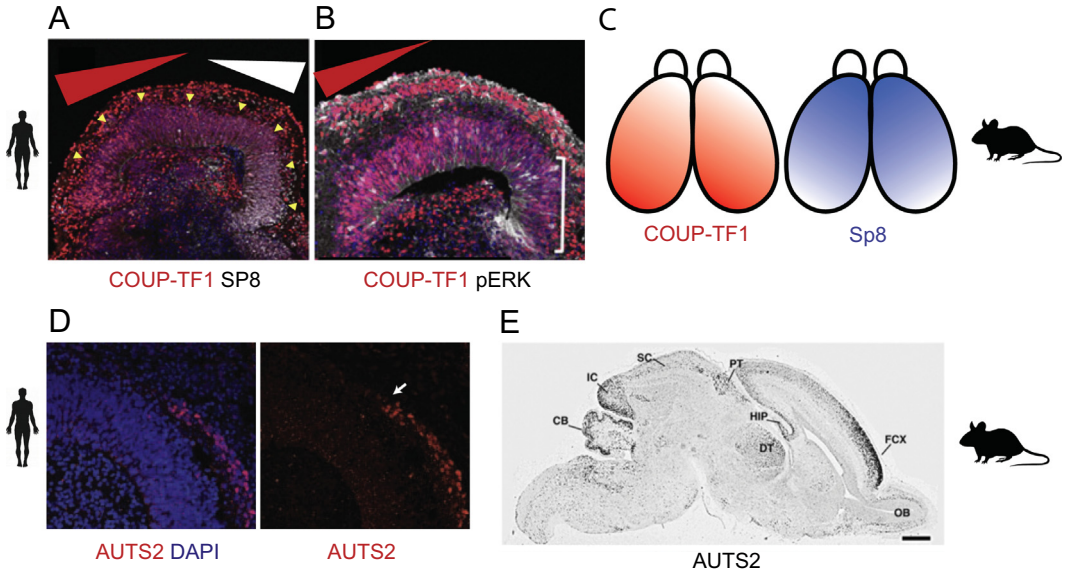

Fig. 5. Gradients of gene expression in cerebral organoids. (A) The transcription factors COUP-TF1 (shown in red) and SP8 (shown in white) are expressed in counter gradients in a section taken from a human cerebral organoid. (B) Expression of COUP-TF1 and pERK (white bracket) in a human cerebral organoid) is highest in the region where COUP-TF1 expression is lowest, indicating that FGF signaling is regionally active and could underlie the transcription factor expression gradients. (C) Schematic showing high postero-lateral to low antero-medial and high antero-medial to low postero-lateral gradients shown respectively by COUP-TF1 and SP8 expression in mouse cortex *in vivo*. (D) The transcription factor AUTS2 is expressed regionally in human organoids, consistent with its strong enrichment in the frontal cortex (FCX) region of the late-gestation (E19) mouse embryo, as shown in panel (E). Abbreviations: FCX: frontal cortex, OB olfactory bulb, HIP hippocampus, PT prethalamus, SC superior colliculus, IC inferior colliculus, CB cerebellum. Sources: (A) Kadoshima et al. (2013), (B) O'Leary et al. (2007), (C) Lancaster et al. (2013) (D) Bedogni et al. (2010) with permission. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A recent report described a detailed comparison of the transcriptomes of over 300 single cells isolated from human iPSC-derived cerebral organoids at a range of ages (33–65 days in culture) with those of a similar number of individual cells isolated from the cortices of human fetuses at 12–13 PCW (Camp et al., 2015). The organoids contained cells whose transcriptomes matched well with those of apical progenitors, intermediate progenitors and cortical neurons at various stages of differentiation. Both organoids and fetal cortices contained cells whose transcriptomes indicated that they were in the process of transition between stages, consistent with differentiation being a continuous process. When expression levels of the key transcription factors *SOX2*, *TBR2* and *MYT1L* in organoid-derived cortical progenitor and neuronal cells were plotted against the levels found in equivalent cell types isolated from fetal cortex, correlation factors greater than 0.9 were found, indicating a very close correspondence. These strong similarities between organoid and embryo-derived cell transcriptomes is perhaps the best evidence that we have so far that the differentiation programs followed by cerebral organoids *in vitro* match closely those followed by cortical cells *in vivo* (Camp et al., 2015). Sequencing the transcriptomes of single cells from embryonic cortices should be a highly effective way to identify the full set of progenitor cell types present in the developing cortex *in vivo* and subsequently to find out whether or not each type is present in cerebral organoids.

The electrophysiological properties of neurons formed in cerebral organoids derived from human PSCs have also been investigated (Lancaster et al., 2013; Paşca et al., 2015). Neurons in human iPSC-derived cerebral organoids after 75 days in culture exhibited spontaneous Ca^{2+} surges, whose frequency increased in response to added glutamate, indicating the presence of electrically-active glutamatergic cells (Lancaster et al., 2013). Further, Paşca et al. (2015) found clear evidence of functional synapses in organoids after 180 days of culture. They found large amplitude excitatory post-synaptic potentials in response to electrical stimulation, indicating the presence of networks of glutamatergic neurons. It therefore seems clear that fully differentiated, electrically active neurons arise in cerebral organoids and that they are able to form functional synapses. However, given that organoids lack the ventrally-born GABA-ergic interneurons that are required for normal circuit formation in the embryonic cortex, there will be important differences between circuits in organoids and those in the embryonic brain.

Perhaps the most surprising discovery from this work so far is the remarkable extent to which PSCs can recapitulate cortical development in the absence of external signals – i.e. that so much of the program of cortical differentiation appears to be cell-intrinsic. Accordingly, mouse and human PSCs follow appropriate species-specific timelines of differentiation, as described above. Similarly, organoids grown from other primate iPSCs showed species-specific behaviors (Otani et al., 2016). Species-specific behaviors continue into later stages of cortical development, as shown when human iPSC-derived neurons are transplanted into mouse forebrain, where they take several months to elaborate dendritic arbors fully, whereas transplanted neurons derived from mouse PSCs fully arborize in a few weeks (reviewed

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by Anderson and Vanderhaeghen, 2014; Suzuki and Vanderhaeghen, 2015).

CEREBRAL ORGANOIDS AS TOOLS TO UNDERSTAND FOREBRAIN DEVELOPMENT AND DISEASE

Clearly, there is now considerable evidence in support of the idea that cerebral organoids model key aspects of early development of the cerebral cortex in a species-specific manner. It therefore seems likely that they represent a good model system to study normal development of the forebrain in both mice and humans and to understand the basis of neurodevelopmental diseases. Most current studies aimed at understanding the molecular mechanisms that govern embryonic development of the forebrain involve the use of genetically modified animals, designed to investigate the roles of specific genes. It is relatively easy to introduce genetic changes to PSCs. In particular, the advent of CRISPR/Cas9 technology makes it straightforward to generate precise mutations in PSC genomes (Doudna and Charpentier, 2014). Multiple modifications can be made to the same cells – as many as five separate genes have been inactivated simultaneously in mouse ES cells using this method (Wang et al., 2013). Thus, multiple alleles, such as a floxed allele, a cre recombinase transgene and a fluorescent reporter could readily be combined. For mouse studies, this contrasts sharply with the generations of breeding required to create mutant lines carrying suitable combinations of multiple mutant alleles. Gain-of-function, loss-of function or conditional alleles can all be used to investigate the roles played by specific genes at specific stages of cortical development. Given that mouse and human cerebral organoids show multiple species-specific behaviors, as outlined above, it seems likely that they will prove to be useful tools to explore the mechanisms underlying differences between mouse and human forebrain development.

Making cerebral organoids from such iPSCs represents a powerful potential new tool to investigate the developmental mechanisms underlying specific neurodevelopmental disorders, whether or not the gene (s) that are altered in affected individuals have been identified (Marchetto and Gage, 2014). One likely key advantage in using human organoids to unravel neurodevelopmental disease mechanisms is that some such diseases have been difficult to reproduce in mutant mice. For example, mice lacking the *doublecortex* (*dcx*) gene do not show the cortical lamination mutant phenotypes found in humans with *DCX* mutations (Corbo et al., 2002). The effectiveness of an organoid-based approach to studying human neurodevelopmental disorders was demonstrated very effectively by Lancaster et al. (2013) who derived iPSCs from a microcephalic patient who had a mutation in the *CDK5RAP2* gene, then cultured cerebral organoids from the patient-derived cells. These organoids contained fewer actively proliferating progenitor cells than controls and showed premature neural differentiation, suggesting that neural progenitors lacking CDK5RAP2 activity stop proliferating and start to differentiate earlier than normal, leading to formation of smaller cerebral organoids and suggesting a plausible mechanism underlying the microcephalic phenotype (Lancaster et al., 2013). The authors further showed that the mutant phenotype could be rescued by forcing expression of CDK5RAP2 in the mutant iPSCs.

In another recent study, cerebral organoids grown from patient-specific iPSCs were used to investigate the neurodevelopmental abnormalities that underlie idiopathic autism spectrum disorders (Mariani et al., 2015). The authors of this study reported that GABA-ergic inhibitory interneurons were overproduced in organoids derived from patient-specific iPSCS. Examination of the transcriptomes of these organoids suggested that overexpression of the transcription factor FOXG1 was likely to be driving the over-production of GABA-ergic neurons and may be an important contributor to autism spectrum disorders (Mariani et al., 2015).

A neat illustration of the utility of cerebral organoids is provided by a recent cluster of papers from several groups investigating the connection between Zika virus (ZIKV) infection and microcephaly, which is obviously extremely difficult to investigate directly in infected patients. Garcez et al. (2016) infected human iPSC-derived brain organoids with ZIKV and found that infected organoids were 40% smaller compared to controls after 11 days in culture. Qian et al. (2016) found that ZIKV infection led to increased cell death and reduced proliferation in human cerebral organoids grown in innovative miniaturized spinning bioreactors. Cugola et al. (2016) infected human PSC-derived cerebral organoids with ZIKV and found a significant decrease in the number of PAX6-expressing neural progenitor cells and differentiated neurons in infected organoids, most likely as a result of increased cell death. Similarly, Dang et al. (2016) used human ESC-derived cerebral organoids to investigate the pathogenicity of ZIKV. They found that ZIKV efficiently infected progenitor cells, leading to significantly smaller organoids as a consequence of upregulation of the innate immune receptor Toll-like-receptor 3 (TLR3) gene, leading to disrupted neural differentiation and increased cell death. TLR3 has previously been shown to have a negative effect on neural precursor cell proliferation in mouse embryos (Lathia et al., 2008). Interestingly, Nowakowski et al. (2016) used organoids derived from human PSCs to show that oRG express the candidate ZIKV receptor AXL at very high levels, and are therefore likely targets for ZIKV infectivity. Given the importance of oRG in generating cortical neurons in humans, it is easy to see how this could have a large effect on cortical growth. Other cortical cell types, including radial glia also express AXL and it is not yet clear exactly which progenitor subtypes are susceptible to ZIKV infection (Nowakowski et al., 2016). Although preliminary, these studies clearly illustrate the value of cerebral organoids as models for understanding the pathogenicity of ZIKV infection.

In the ten years since iPSC technology was first established (Takahashi and Yamanaka, 2006), studies of the properties of human iPSCs have shown that there can be considerable variation in behavior between iPSC lines, even when derived from the same individual

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(Marchetto and Gage, 2012; Brennand et al., 2015). Therefore it is important to establish that phenotypes observed in patient-specific lines are truly due to the particular mutation, and not to another source of variation. One very effective way to do this is to use CRISPR/Cas9 techniques to correct patient-specific mutations in individual iPSC lines. Thus, a combination iPSC and CRISPR/Cas9 technologies may be particularly useful in uncovering the mechanisms underlying neurodevelopmental diseases.

LIMITATIONS OF CEREBRAL ORGANOIDS AS MODELS OF FOREBRAIN DEVELOPMENT

The evidence summarized above indicates that cerebral organoids represent a good approximation to early stages of cerebral cortex development *in vivo*. However, some important differences remain, likely as a result of limitations to the existing culture methods. For example, organoids fail to develop the clear lamination pattern found in embryonic cerebral cortex, suggesting that radial migration of newborn cortical neurons does not occur as normal (Nasu et al., 2012; Lancaster et al., 2013; Kadoshima et al., 2013). At present, therefore, organoids are likely to be most useful for studies of early cortical development. However, cerebral organoid technology is in its infancy, and it is likely that refinements to the existing protocols will enable more accurate modeling of cortical development, including its later stages.

Most probably, the differences between organoids and embryonic brains arise from differences in the environments in which they develop. Clearly, *in vivo*, the cortex does not develop in isolation, it is surrounded by other tissues which affect its development. These include blood vessels, the meninges (a specialized membrane that surrounds the developing brain and which releases diffusible signals that affect cell proliferation and differentiation Siegenthaler and Pleasure (2011)) and the ganglionic eminences, from which the GABAergic inhibitory neurons required for cortical circuitry emerge and subsequently migrate into the developing cortex. The lack of vascularization has obvious consequences for gas exchange, nutrient supply and waste product removal as organoids get larger, but culturing organoids in a spinning bioreactor (Lancaster et al., 2013) or in the presence of high O_2 levels (Kadoshima et al., 2013) may compensate for this. Future refinements to organoid differentiation protocols could be designed to generate organoids that include both cortical tissue and ganglionic eminences adjacent to one another, as in the embryo. One possible way to do this could involves the localized application of specific signaling molecules, perhaps using fluid engineering techniques, to allow growth and patterning of cerebral organoids that more closely resemble normal brain tissues. Along these lines, addition of Shh agonists to organoid cultures promoted the formation of Gsx2-expressing ventral telencephalic tissue that abutted areas of Pax6-expressing cortical neuroepithelium, as is normally seen at the boundary between the cortex and the ganglionic eminences *in vivo* (Kadoshima et al., 2013).

There is heterogeneity in the efficiency with which current protocols produce organoids that resemble embryonic cortex (Nasu et al., 2012; Mariani et al., 2012; Lancaster et al., 2013; Kadoshima et al., 2013). One likely cause of such heterogeneity is the use of Matrigel, a commercially available form of extracellular matrix used in organoid differentiation protocols. Matrigel is purified from tumor material and its precise composition varies from batch to batch (Kleinman and Martin, 2005). It is possible to substitute for Matrigel using synthetic, defined matrices, indicating that its scaffolding properties are needed, rather than any effect of growth factors or other proteins that it may contain (Meinhardt et al., 2014; Lindborg et al., 2016). However, the extent to which existing synthetic matrices can completely replace Matrigel for the preparation of cerebral organoids has not yet been fully established.

CONCLUSION

Cerebral organoids present an exciting new tool to help us explore mechanisms of brain development in mammals and the underlying causes of neurodevelopmental diseases in man. They should allow us to characterize the normal behaviors of each of the increasingly large number of progenitor cell types found in the human embryonic cortex, to decipher the genetic mechanisms that regulate these behaviors and, ultimately, to understand exactly how dysregulation of these mechanisms can lead to specific neurodevelopmental diseases.

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